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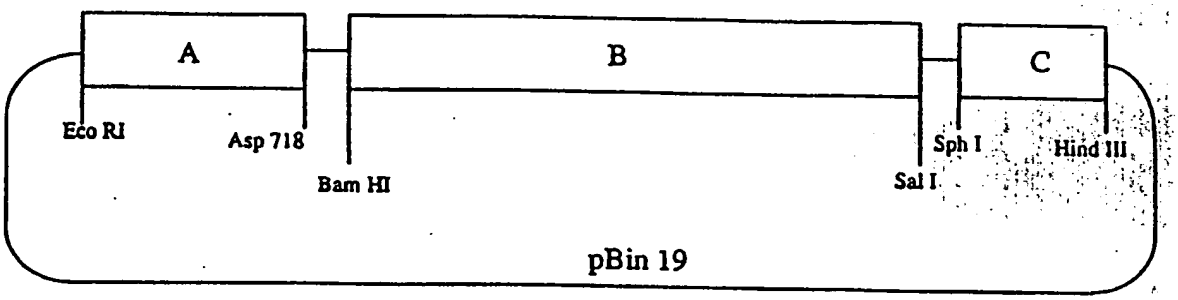
(54) Title: DNA SEQUENCES WHICH LEAD TO THE FORMATION OF POLYFRUCTANS (LEVANS), PLASMIDS CONTAINING THESE SEQUENCES AS WELL AS A PROCESS FOR PREPARING TRANSGENIC PLANTS

p35S-CW-LEV:



(57) Abstract

There are described DNA sequences, which lead to the formation of polyfructans (levans), plasmids containing theses DNA sequences, as well as a process using these plasmids for preparing transgenic plants with polyfructan (levan) expression.

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Title: DNA sequences which lead to the formation of polyfructans (levans), plasmids containing these sequences as well as a process for preparing transgenic plants

Field of the invention

The present invention relates to DNA sequences which lead to the formation of polyfructans (levans), as well as a process for preparing transgenic plants using plasmids on which these DNA sequences are located.

High molecular weight, water soluble, linear polymers, for example those based on polyacrylates or polymethacrylates, are products of mineral oils and have many important uses. In particular their properties in increasing viscosity of aqueous systems, in suspending or sedimentation acceleration and complexing are especially valuable from the technical viewpoint. These products are also used in exceptionally large amounts in super absorbers for water binding and in water dilutable lacquers. In spite of the outstanding positive properties, because such products are difficult to dispose of, their use is increasingly coming under criticism because they are not biodegradable.

Alternatives based on recyclable raw materials, especially starches and cellulose, because of the macromolecular structure of these polysaccharides, have been shown to have limited value. As a replacement for non-biodegradable chemically derived polymers, a number of derivatised high polymeric polysaccharides have been considered. Until now, such polysaccharides could only be obtained biotechnologically via suitable fermentation and transglycosidation processes. The products obtained in this way, such as dextrans and polyfructans (levans) are

not competitive as raw materials for mass production.

Polyfructans are found in a number of monocotyledonous and dicotyledonous higher plants, in green algae as well as in a number of gram positive and gram negative bacteria (Meier and Reid, (1982) Encyclopedia of Plant Physiology, New Series, 13A. 418 - 471). The role of fructans for the plant development and plant growth is not fully understood. Functions of the fructans that have been proposed are as a protection against freezing at low temperatures, as alternative carbohydrate stores by limiting starch biosynthesis, as well as applied intermediary stores for photoassimilates, situated in the stems of grasses, shortly before their transfer into the seeds.

All fructans contain as starter molecule for the polymerisation reaction, a molecule of sucrose (glucose-fructose) to which fructose polymers are added.

Depending on the coupling of the fructose molecule, fructans of plant origin can be classified into four classes (Meier and Reid (1982), Encyclopedia of Plant Physiology, New Series, 13A, 418 - 471):

a) (2-1) coupled β-D-fructans (inulin type)

b) (2-6) coupled β-D-fructans (phlein or levan type)

c) highly branched fructans with a mixture of 2-1 and 2-6 couplings.

d) (2-1) coupled β-D-fructans, which in contrast to the types under a - c, are added completely from fructose residues of polymerisation both from glucose

and also from fructose residues from polyfructose residues (neokestose type).

Fructans of bacterial origin correspond either to the levan or to the inulin type (Carlsson (1970) Caries Research 4, 97 - 113) and Dedonder (1966) Methods Enzymology 8, 500 - 505).

Experiments on the biosynthesis of fructans in plants and bacteria lead one to conclude that this proceeds by various routes. Bacterial and plant fructans are further distinguished, not particularly in their primary structure but mainly in their molecular weight. Thus, fructans isolated from plants have been shown to have molecular weights of between 5000 and 50,000 d (Pollock and Chatterton (1988) in: The Biochemistry of Plants 14, 109 - 140), whilst for fructans isolated from bacteria, molecular weights of up to 2,000,000 d have been described (Clarke et al (1991) in: Carbohydrates as Organic Raw Materials, VCH Weinheim, 169 - 182).

Various microorganisms from the group of *Bacillus* spp as well as *Streptococcus* spp produce polyfructoses in which both fructans of the levan type and fructans of the inulin type have been described (Carlsson (1970) Caries Research 4, 97 - 113 and Dedonder (1966) Methods Enzymology 8, 500 - 505).

Experiments on biosynthesis pathways have made it clear that, in comparison to biosynthesis pathways in higher plants, there is a more simple pattern and a sharing of only one enzyme. This enzyme with the trivial name levan sucrase is a transfructosylase (sucrose:ß-D-fructosyl transferase, E.C.2.4.1.10.), which catalyses the following reaction:

sucrose + acceptor ⇒ glucose + fructosyl acceptor

Representative acceptors are water, alcohol, sugar or polyfructoses. The hypothesis that only one enzyme catalyses this reaction, depends on the one hand on the examination of the protein chemically purified enzyme, and on the other to the fact that the gene for levan sucrase has been isolated from various *Bacillus* spp. as well as from a *Streptococcus* spp. and after transfer into *E. coli* leads to the formation of levan in *E. coli* (Gay et al (1983) J. Bacteriology 153, 1424 - 1431 and Sato et al. (1986) Infection and Immunity 52, 166 - 170).

Until now, genes for levan sucrase from *Bacillus amyloliquefaciens* (Tang et al. (1990) Gene 96, 89 - 93) and *Bacillus subtilis* (Steinmetz et al. (1985) Mol. Gen. Genetics 200, 220 - 228), have been described, which demonstrate relatively high homology with each other and both of which catalyse the synthesis of fructans of the levan type. Further a fructosyl transferase from *Streptococcus mutans* (Shiroza et al. (1988) J. Bacteriology 170, 810 - 816) has been described. This shows little homology to either levan sucrases from *Bacillus* spp.. The fructan formed in *Streptococcus mutans* is of the inulin type.

In WO 89/12386, there is described the possibility of producing carbohydrate polymers such as dextran or levan in transgenic plants, especially in the fruit of transgenic plants. To prepare these plants, the use of levan sucrases from *Aerobacter levanicum*, *Streptococcus salivarius* and *Bacillus subtilis* and the use of dextran sucrases from *Leuconostoc mesenteroides* have been described.

Further the construction of chimeric genes is described which may be suitable for the expression of the levan sucrase from *Bacillus subtilis* as well as the dextran sucrase from *Leuconostoc mesenteroides* in transgenic plants. Also described is the preparation of transgenic plants containing these constructs. Further, the preparation of transgenic plants that contain these constructs are described. Whether polyfructans can actually be produced by the described process is not known.

There is also described a series of processes for modifying the carbohydrate concentration and/or concentrating carbohydrate in transgenic plants by means of biotechnological methods. Thus, in view of the fact that increasing starch concentration and modification of the starch in physical and chemical respects is already known, then a modification of the carbohydrate content of potato plants by raising or lowering the ADP-glucose-pyrophosphorylase activity can be achieved (EP 455 316).

From EP 442 592 it is further known that a modification of the distribution of photoassimilates by means of cytosolic and apoplastic invertase is possible and that the yield as well as the drought and frost resistance of potato plants through expression of a heterologous pyrophosphatase gene in potato plants can be modified.

In order to adapt the physico-chemical parameters of raw materials which are increasingly being used, such as polysaccharides, to the requirements of the chemical industry, as well as to minimise the costs of obtaining these products, processes for the preparation of transgenic plants have to be developed which lead in comparison with known processes to better, higher yielding plants.

It has now been surprisingly found that the DNA sequence of the levan sucrase from a gram-negative bacterium of the species *Erwinia amylovora* with the nucleotide sequence (Seq - ID NO 1):

```
GGATCCCCCG GCTGCAGCG ATCATGGTTA TTTATAAGGG ATTGTTATGT          50
CCTGAAAACC ACACAACAGA ACCAGAGTGA TTTCAAAAAA TAAAAAGCTA        100
TTAATATACA GACCTTCAGC AAGAAGGTAT TCGAAATAAC CTGTGAGGAT
                                                              150
ATTT  ATG TCA GAT
      Met Ser Asp                                             163

TAT AAT TAT AAA CCA ACG CTG TGG ACT CGT GCC GAT GCA TTG AAA
Tyr Asn Tyr Lys Pro Thr Leu Trp Thr Arg Ala Asp Ala Leu Lys   208
  5                  10                  15
GTT CAT GAG GAT GAC CCA ACC ACA ACT CAA CCG GTT ATT GAC ATT   253
Val His Glu Asp Asp Pro Thr Thr Thr Gln Pro Val Ile Asp Ile
 20                  25                  30
GCA TTC CCG GTA ATG AGT GAA GAA GTC TTT ATT TGG GAT ACC ATG
Ala Phe Pro Val Met Ser Glu Glu Val Phe Ile Trp Asp Thr Met   298
 35                  40                  45
CCA TTG CGA GAC TTC GAC GGA GAG ATT ATC TCT GTA AAT GGT TGG
Pro Leu Arg Asp Phe Asp Gly Glu Ile Ile Ser Val Asn Gly Trp   333
 50                  55                  60
TGT ATT ATT TTT ACG CTA ACA GCA GAT CGC AAC ACT GAT AAT CCG   388
Cys Ile Ile Phe Thr Leu Thr Ala Asp Arg Asn Thr Asp Asn Pro
 65                  70                  75
CAA TTC CAG GAT GAA AAT GGC AAT TAT GAT ATT ACT CGT GAC TGG
Gln Phe Gln Asp Glu Asn Gly Asn Tyr Asp Ile Thr Arg Asp Trp   433
 80                  85                  90
GAA GAC AGA CAT GGT CGT GCG CGT ATT TGT TAT TGG TAC TCA CGC   478
Glu Asp Arg His Gly Arg Ala Arg Ile Cys Tyr Trp Tyr Ser Arg
 95                 100                 105
ACC GGT AAA GAC TGG ATT TTT GGC GGT CGG GTA ATG GCC GAA GGT   523
Thr Gly Lys Asp Trp Ile Phe Gly Gly Arg Val Met Ala Glu Gly
110                 115                 120
GTC GCA CCG ACG ACG CGT GAG TGG GCC GGA ACC CCG ATC CTT TTA
Val Ala Pro Thr Thr Arg Glu Trp Ala Gly Thr Pro Ile Leu Leu   568
125                 130                 135
AAC GAT CGG GGC GAT ATT GAC CTG TAT TAT ACC TGT GTC ACT CCG   613
Asn Asp Arg Gly Asp Ile Asp Leu Tyr Tyr Thr Cys Val Thr Pro
140                 145                 150
GGT GCA ACC ATT GCC AAA GTG CGC GGT AAA ATC GTC ACT TCC GAT   658
Gly Ala Thr Ile Ala Lys Val Arg Gly Lys Ile Val Thr Ser Asp
155                 160                 165
CAA AGT GTA AGC CTG GAA GGT TTT CAG CAG GTT ACA TCA CTT TTC
Gln Ser Val Ser Leu Glu Gly Phe Gln Gln Val Thr Ser Leu Phe   703
170                 175                 180
TCT GCT GAC GGG ACT ATT TAC CAG ACG GAA GAG CAG AAC GCT TTC   748
Ser Ala Asp Gly Thr Ile Tyr Gln Thr Glu Glu Gln Asn Ala Phe
185                 190                 195
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TGG AAC TTC CGT GAC CCA AGC CCA TTC ATT GAC AGG AAT GAT GGC
Trp Asn Phe Arg Asp Pro Ser Pro Phe Ile Asp Arg Asn Asp Gly  793
    200                 205                 210
AAA TTA TAT ATG CTG TTT GAA GGA AAC GTG GCG GGG CCG CGC GGT
Lys Leu Tyr Met Leu Phe Glu Gly Asn Val Ala Gly Pro Arg Gly  838
    215                 220                 225
TCG CAC GAA ATT ACC CAG GCT GAG ATG GGT AAT GTG CCG CCG GGT  883
Ser His Glu Ile Thr Gln Ala Glu Met Gly Asn Val Pro Pro Gly
    230                 235                 240
TAT GAA GAT GTG GGT GGC GCA AAA TAT CAG GCA GGC TGT GTT GGT
Tyr Glu Asp Val Gly Gly Ala Lys Tyr Gln Ala Gly Cys Val Gly  928
    245                 250                 255
CTG GCT GTG GCC AAA GAC CTG TCA GGC AGT GAG TGG CAA ATC CTG
Leu Ala Val Ala Lys Asp Leu Ser Gly Ser Glu Trp Gln Ile Leu  973
    260                 265                 270
CCT CCG CTG ATC ACC GCT GTT GGC GTA AAC GAT CAG ACT GAA CGC 1018
Pro Pro Leu Ile Thr Ala Val Gly Val Asn Asp Gln Thr Glu Arg
    275                 280                 285
CCT CAT TTT GTC TTC CAG GAT GGT AAA TAC TAT CTG TTC ACC ATT
Pro His Phe Val Phe Gln Asp Gly Lys Tyr Tyr Leu Phe Thr Ile 1063
    290                 295                 300
AGC CAT AAG TAC ACT TTT GCC GAT AAC CTG ACC GGC CCT GAT GGA 1108
Ser His Lys Tyr Thr Phe Ala Asp Asn Leu Thr Gly Pro Asp Gly
    305                 310                 315
GTG TAT GGC TTT GTA AGC GAT AAA CTT ACC GGC CCT TAC ACG CCG
Val Tyr Gly Phe Val Ser Asp Lys Leu Thr Gly Pro Tyr Thr Pro 1153
    320                 325                 330
ATG AAT AGC TCC GGG CTG GTG CTG GGC AAC CCG TCT TCA CAA CCT 1198
Met Asn Ser Ser Gly Leu Val Leu Gly Asn Pro Ser Ser Gln Pro
    335                 340                 345
TTC CAG ACA TAT TCA CAC TAT GTT ATG CCT AAT GGG CTG GTC ACT 1243
Phe Gln Thr Tyr Ser His Tyr Val Met Pro Asn Gly Leu Val Thr
    350                 355                 360
TCC TTT ATT GAC AGT GTT CCG TGG AAA GGT AAG GAC TAT CGC ATT
Ser Phe Ile Asp Ser Val Pro Trp Lys Gly Lys Asp Tyr Arg Ile 1288
    365                 370                 375
GGC GGT ACT GAA GCT CCG ACC GTA AAA ATT CTG TTG AAA GGC GAT
Gly Gly Thr Glu Ala Pro Thr Val Lys Ile Leu Leu Lys Gly Asp 1333
    380                 385                 390
CGC TCA TTT ATT GTT GAT AGC TTC GAT TAT GGA TAT ATT CCG GCA 1378
Arg Ser Phe Ile Val Asp Ser Phe Asp Tyr Gly Tyr Ile Pro Ala
    395                 400                 405
ATG AAA GAC ATT ACT TTA AAA TAAGTCTGTT GTCGATATCA AGCTTATCGA 1429
Met Lys Asp Ile Thr Leu Lys
    410                 415
TACCGTCGA  1438
```

makes possible the preparation of large amounts of polyfructans (levans) in transgenic plants, which decisively meet the needs of the chemical industry in respect of recyclable raw materials.

By integration of a DNA sequence in a plant genome, on which the above given DNA sequence is located, the polyfructan (levan), expression in plants, especially in leaves and tubers is made possible. The levan sucrase of the invention shows, at the DNA level, no significant homology to the known levan sucrases.

The invention further provides a process for the preparation of transgenic plants with polyfructan (levan) expression in leaves and tubers that comprises the following steps:

(a) preparation of a DNA sequence with the following partial sequences:
  i) a promoter which is active in plants and ensures formation of an RNA in the intended target tissues or target cells,
  ii) a DNA sequence of a levan sucrase, and
  iii) a 3'-non-translated sequence, which in plant cells leads to the termination of the transcription as well as the addition of poly A residues to the 3'-end of the RNA,
(b) transfer and integration of the DNA sequence in the plant genome of a recombinant double stranded DNA molecule from plant cells using a plasmid, and
(c) regeneration of intact whole plants from the transformed plant cells.

The levan sucrose obtained in process step (a,) ii) preferably shows the nucleotide sequence noted under sequence IC No 1.

The levan sucrase catalyses the following reaction:

Sucrose-$(fructose)_n$ + sucrose ⇒ sucrose-$(fructose)_{n+1}$ + glucose.

Using this process in principle, all plants can be modified in respect to a polyfructan (levan) expression, preferably crops such as maize, rice, wheat, barley, sugar beet, sugar cane, tobacco and potatoes.

In process step (b), in principle all plasmids can be used which have the DNA sequence given under sequence ID No 1. Preferably used are plasmid p35s-CW-LEV (DSM) 7186), plasmid P35s-CY-LEV (DSM 7187) or plasmid P33-CW-LEV (DSM 7188).

Since sucrose represents the substrate for the levan sucrase, the production of polyfructans is especially advantageous in those organs which store large amounts of sucrose. Such organs are for example the roots of sugar beet or the stems of sugar cane. It is especially useful in genetically modified potatoes, which store sucrose in their tubers, through blocking of starch biosynthesis

Biosynthesis of sucrose takes place in the cytosol, whilst in contrast, storage is in the vacuole. During transport into the storage tissues of sugar beet or potato or into the endosperm of seeds, the sucrose must cross the intercellular space. In production of polyfructans, all three cell compartments are suitable, i.e. cytosol, vacuole and intercellular space.

The coding sequence of the levan sucrose of the nucleotide sequence ID No 1 can be provided with a promoter that ensures the transcription in specified orders which is coupled in sense orientation (3'-end of the promoter to the 5'-end of the coding sequence) on the coding sequence

which codes the enzyme to be formed. The termination signal which determines the termination of the mRNA synthesis is adhered to the 3'-end of the coding sequence. In order to direct the enzyme which is expressed in specified sub-cellular compartments such as chloroplasts, amyloplasts, mitochondria, vacuoles, cytosol or intercellular space, a so-called signal sequence or a transit peptide coding sequence can be positioned between the promoter and the coding sequence. This sequence must be in the same reading frame as the coding sequence of the enzyme.

For the introduction of the DNA sequence of the invention in higher plants, a large number of cloning vectors are available, which contain a replication signal for *E. coli* and a marker, which allows a selection of the transformed cells. Examples of vectors are pBR 322, pUC-series, M13 mp-series, pACYC 184; EMBL 3 etc.. According to the introduction method of the desired gene in the plant, other DNA sequences may be suitable. Should the Ti- or Ri-plasmid be used, e.g. for the transformation of the plant cell, then at least the right boundary, often however both the right and left boundary of the Ti- and Ri-Plasmid T-DNA, is attached, as a flanking region, to the gene being introduced. The use of T-DNA for the transformation of plants cells has been intensively researched and is well described in EP 120 516; Hoekama, In: The Binary Plant Vector System, Offset-drukkerij Kanters B.V. Alblasserdam, (1985), Chapter V; Fraley, et al., Crit. Rev. Plant Sci., 4:1-46 and An et al. (1985) EMBO J. 4: 277-287. Once the introduced DNA is integrated in the genome, it is as a rule stable there and remains also in the offspring of the original transformed cells. It normally contains a selection marker, which induces resistance in the transformed plant cells against a

biocide or antibiotic such as kanamycin, G 418, bleomycin, hygromycin or phosphinotricin etc. The individual marker employed should therefore allow the selection of transformed cells from cells, which lack the introduced DNA.

For the introduction of DNA into a plant, besides transformation using *Agrobacteria*, there are many other techniques available. These techniques include the fusion of protoplasts, microinjection of DNA and electroporation, as well as ballistic methods and virus infection. From the transformed plant material, whole plants can be regenerated in a suitable medium, which contains antibiotics or biocides for the selection. The resulting plants can then be tested for the presence of introduced DNA. No special demands are placed on the plasmids in injection and electroporation. Simple plasmids, such as e.g. pUC-derivatives can be used. Should however whole plants be regenerated from such transformed cells the presence of a selectable marker gene is necessary. The transformed cells grow within the plants in the usual manner (see also McCormick et al.(1986) Plant Cell Reports 5: 81-84). These plants can be grown normally and crossed with plants, that possess the same transformed genes or different. The resulting hybrid individuals have the corresponding phenotypical properties.

Deposits

The following plasmids were deposited at the Deutschen Sammlung von Mikroorganismen (DSM) in Braunschweig, Germany on the 16.07.1992 (deposit number):

Plasmid p35s-CW-LEV (DSM 7186)
Plasmid p35s-CY-LEV (DSM 7187)
Plasmid p33-CW-LEV (DSM 7188)

Description of the Figures

Fig. 1 shows the structure of the p35-CW-LEV plasmid. It comprises the three fragments A, B and C.
Fragment A contains the 35s promoter of the cauliflower mosaic virus (CaMV), nucleotides 6906 - 7437.
Fragment B contains the sequence of the nucleotides 689 - 2122 of the levan sucrase from *Erwinia amylovora* (Seq. ID No.1).
Fragment C contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid, pTi ACH 5, nucleotides 11749 - 11939.

Fig. 2 shows the structure of the p35s-CY-LEV plasmid. It comprises the three fragments A, B and C.
Fragment A contains the 35s promoter of the cauliflower mosaic virus (CaMV), nucleotides 6909 - 7437.
Fragment B contains the sequence of the nucleotides 864 - 2122 of the levan sucrase from *Erwinia amylovora* (Seq. ID No.1).
Fragment C contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid, pTi ACH 5.

Fig. 3 shows the structure of the p33-CW-LEV plasmid. It comprises the three fragments A, B and C.
Fragment A contains the DraI-DraI-fragment (position -1512 to position +14) of the promoter region of the patatin gene B33.
Fragment B contains the sequence of the nucleotides 689 - 2122 of the levan sucrase from *Erwinia amylovora* (Seq. ID No.1).
Fragment C contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid,

pTi ACH 5, nucleotides 11749 - 11939.

Fig. 4 shows the detection of polyfructan in transformed tobacco plants (No. 2, 3 and 13). In this:
Fru = fructose, Suc = sucrose, Kes = kestose
c1 = control 1, c2 = controle 2, M = marker

In order to understand the examples forming the basis of this invention all the processes necessary for these tests and which are known per se will first of all be listed:

1. Cloning process

The vector pUC 18 (Yanisch-Perron et al. (1985) Gene 33: 103-119) was used for cloning.

For the plant transformations, the gene constructs were cloned in the binary vector BIN 19 (Bevan (1984) Nucl. Acids Res 12: 8711-8720)

2. Bacterial strains

The *E. coli* strain BMH71-18 (Messing et al., Proc. Natl. Acad. Sci. USA (1977), 24, 6342-6346) or TB1 was used for the pUC vectors. TB1 is a recombinant-negative, tetracycline-resistant derivative of strain JM101 (Yanisch-Perron et al., Gene (1985), 33, 103-119). The genotype of the TB1 strain is (Bart Barrel, personal communication): F'(traD36, proAB, lacI, lacZΔM15), Δ(lac, pro), SupE, thiS, recA, Sr1::Tn10(TcR).

The transformation of the plasmids into the potato plants was carried out using *Agrobacterium tumefaciens* strain LBA4404 (Bevan, (1984), Nucl. Acids Res. 12, 8711-8720).

3. Transformation of *Agrobacterium tumefaciens*

In the case of BIN19 derivatives, the insertion of the DNA into the *Agrobacterium* was effected by direct transformation in accordance with the method of Holsters et al., (1978) (Mol Gene Genet 163: 181-187). The plasmid DNA of the transformed *Agrobacterium* was isolated in accordance with the method of Birnboim and Doly (1979) (Nucl Acids Res 7: 1513-1523) and was analysed by gel electrophoresis after suitable restriction cleavage.

4. Plant transformation

A) Tobacco: 10 ml of an overnight culture of *Agrobacterium tumefaciens*, grown under selection, were centrifuged off, the supernatant was discarded, and the bacteria were resuspended in the same volume of antibiotic-free medium. In a sterile petri dish, leaf discs of sterile plants (approximately 1 cm^2), the central vein of which had been removed, were immersed in this bacterial suspension. The leaf discs were then placed in a closely packed arrangement in petri dishes containing MS medium (Murashige et al. (1962) Physiologia Plantarum 15, 473-497) with 2% sucrose and 0.8% bacto agar. After two days incubation in the dark at 25°C, they were transferred onto MS medium containing 100 mg/l kanamycin, 500 mg/l claforan, 1 mg/l benzylaminopurine (BAP), 0.2 mg/l of naphthylacetic acid (NAA) and 0.8 % bacto agar. Growing shoots were transferred onto hormone-free MS medium with 250 mg/l of claforan.

B) Potato: Ten small leaves, wounded with a scalpel, of a sterile potato culture were placed in 10 ml of MS medium with 2% sucrose containing 30-50 µl of an *Agrobacterium tumefaciens* overnight culture grown under selection. After 3-5 minutes gentle shaking, the leaves were laid out on MS

medium of 1.6% glucose, 2 mg/l of zeatin ribose, 0.02 mg/l of naphthylacetic acid, 0.02 mg/l of gibberellic acid, 500 mg/l of claforan, 50 mg/l of kanamycin and 0.8% bacto agar. After incubation for one week at 25°C and 3000 lux, the claforan concentration in the medium was reduced by half. Further cultivation was carried out using the method described by Rocha-Sosa et al. (1989) EMBO Journal 8, 29).

5. Analysis of genomic DNA from transgenic plants

The isolation of genomic plant DNA was carried out according to Rogers et al. (1985) Plant Mol Biol 5, 69-76).

For the DNA analysis, after suitable restriction cleavage, 10 to 20 µg of DNA were analysed, by means of Southern blotting, for the integration of the DNA sequences to be investigated.

6. Analysis of the total RNA from transgenic plants

The isolation of plant total RNA was carried out according to Logemann *et al.* (1987), Analytical Biochem. 163, 16-20.

For the analysis, 50 µg portions of total RNA were investigated, by means of Northern blotting, for the presence of the transcripts sought.

7. Extraction and determination of polyfructose in plants

The extraction and determination were carried out according to the method of Portis H. G. (1990), Meth. Plant Biochem. 2, 353-369.

Example 1

Preparation of plasmid p35s-CW-LEV and insertion of the plasmid into the genome of tobacco and potato

The plasmid p35s-CW-LEV comprises the three fragments A, B and C, which were cloned in the cutting sites for restriction enzymes of the polylinker from pUC 18 (see Fig. 1).

Fragment A contains the 35S promoter of cauliflower mosaic virus (CaMV). It contains a fragment that includes the nucleotides 6909 to 7437 of CaMV (Franck *et al.*, (1980) Cell 21, 285-294) and was isolated as Eco RI-Kpn I fragment from plasmid pDH 51 (Pietrzak et al., Nucleic Acids Research 14, 5857-5868) and cloned between the Eco RI-Kpn I cutting sites of the polylinker of plasmid pUC 18.

Fragment B contains the sequence of the nucleotides 689 - 2122 of the gene of the levan sucrase from *Erwinia amylovora* (Seq. ID No.1) and was cloned between the BamHI/SalI cutting positions of the polylinker of pUC 18.

Fragment C contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid, pTi ACH 5 (Gielen et al (1984); EMBO J. 3, 835 - 846) nucleotides 11749 - 11939 which was insolated as Pvu II-Hind III fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209 - 213) and, after addition of Sph I linkers to the Pvu II cutting positions, was cloned between the SphI-Hind III cutting positions of the polylinker of pUC 18.von pUC 18. The plasmid p35s-CW-LEV has a size of 2151 bp.

The part of the plasmid p35s-CW-LEV comprising the fragments A, B and C was introduced in binary vectors and

using the *Agrobakteria* system was introduced into tobacco and potato plants. Intact plants were regenerated from transformed cells. The analysis of the leaves from a series of Tobacco plants transformed with this gene, clearly showed the presence of polyfructan (levan) which is traced back to the expression of the gene 35s-Cw-LEV (see Fig. 4).

Example 2

Preparation of plasmid p35s-CY-LEV and insertion of the plasmid into the genome of tobacco and potato

This Example was carried out in an analogous manner to that described under Example 1, but with the modification, that the Fragment B (coding for the levan sucrase) is shortened on the nucleotide at the 5'-end. This results in the expression of the protein in the cytosol of transgenic plants.

The plasmid p35s-CY-LEV comprises the three fragments A, B and C, which were cloned in the cutting sites for restriction enzymes of the polylinker from pUC 18 (see Fig. 2).

Fragment A contains the 35S promoter of cauliflower mosaic virus (CaMV). It contains a fragment that includes the nucleotides 6909 to 7437 of CaMV (Franck *et al.* (1980) Cell 21, 285-294) and was isolated as Eco RI-Kpn I fragment from plasmid pDH 51 (Pietrzak et al., Nucleic Acids Research 14, 5857-5868) and cloned between the Eco RI-Kpn I cutting sites of the polylinker of plasmid pUC 18.

Fragment B contains the sequence of the nucleotides 864-2122 of the gene of the levan sucrase from *Erwinia*

amylovora (Seq. ID No.1) and was cloned between the SmaI/SalI cutting positions of the polylinker of pUC 18.

Fragment C contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid, pTi ACH 5 (Gielen et al (1984); EMBO J. 3, 835 - 846) nucleotides 11749 - 11939 which was insolated as Pvu II-Hind III fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209 - 213) and, after addition of Sph I linkers to the Pvu II cutting positions, was cloned between the SphI-Hind III cutting positions of the polylinker of pUC 18.von pUC 18. The plasmid p35s-CY-LEV has a size of 1976 bp.

The part of the plasmid p35s-CY-LEV comprising the fragments A, B and C was introduced in binary vectors and using the *Agrobakteria* system was introduced into tobacco and potato plants. Intact plants were regenerated from transformed cells.

Example 3

Preparation of plasmid p35s-CY-LEV and insertion of the plasmid into the genome of tobacco and potato

This Example was carried out in an analogous manner to that described under Example 1, but with the 35s promoter being replaced with the promoter of the class I patatin Gene B33 (Rocha-Sosa et al, (1989) EMBO J 8, 23 - 29) The plasmid p33-CW-LEV comprises the three fragments A, B and C, which were cloned in the cutting sites for restriction enzymes of the polylinker from pUC 18 (see Fig. 3).

Fragment A contains the DraI-DraI fragment (position -1512 to position +14) of the promoter region of the patatin gene B33 (Rocha-Sosa et al (1989) EMBO J. 8, 23 - 29),

which was cloned in the Sma I position of the polylinker of pUC 118.

Fragment B contains the sequence of the nucleotides 689-2122 of the gene of the levan sucrase from *Erwinia amylovora* (Seq. ID No.1) and was cloned between the BamHI/SalI cutting positions of the polylinker of pUC 18.

Fragment C contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid, pTi ACH 5 (Gielen et al (1984); EMBO J. 3, 835 - 846) nucleotides 11749 - 11939 which was insolated as Pvu II-Hind III fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209 - 213) and, after addition of Sph I linkers to the Pvu II cutting positions, was cloned between the SphI-Hind III cutting positions of the polylinker of pUC 18.von pUC 18. The plasmid p33-CW-LEV has a size of 3149 bp.

The part of the plasmid p33-CW-LEV comprising the fragments A, B and C was introduced in binary vectors and using the *Agrobakteria* system was introduced into tobacco and potato plants. Intact plants were regenerated from transformed cells. The analysis of the leaves from a series of Tobacco plants transformed with this gene, clearly showed the presence of polyfructan (levan) which is traced back to the expression of the gene 33-CW-LEV.

Example 4

Analysis of β2,6-D-Fructofurane (levan) synthesised in transgenic plants by 13C-NMR spectroscopy

The analysis of transgenic plants transformed with the construct p35s-CW-LEV is shown as an example. This analysis can equally be applied to transgenic plants transformed with the constructs p35S-CW-LEV or p35s-CY-LEV.

To obtain sufficient amounts of levan synthesised by transgenic plants to perform NMR spectroscopy, about 10g of leave tissue were grinded in 10ml of water. The homogenate is than centrifuged at 4000 Rpm in a Beckman Minifuge and the supernatant is applied to a PD10 column (LKB-Pharmacia) to remove lower molecular weight compounds. The column had been equilibrated with water before 2.5 ml of the supernatant are applied and higher molecular weight compounds are then eluted with 3.5 ml of water. The elute was further purified by adding ion exchange beads (AG 501 X8, Biorad) and shaking for 30 minutes. After centrifugation at 4000 Rpm (Minifuge, Beckman) to remove the beads, the supernatant is applied to a Sepharose 4B column (diameter 16 cm, separating volume 24 ml) to remove short sugar chains. The elute is vacuum dried in a vacuum centrifuge (univapo 150 H, Uniquip, Martinsried (FRG) and than analysed by 13C-NMR under the following conditions:

PULPROG	zgdc30	F2 - Processing parameters	
SOLVENT	D20	SI	32768
AQ	1.3762726 sec	SF	100.5485322 MHz
FIDRES	0.363305 Hz	WDW	EM
DW	21.0 usec	SSB	0
RG	32768	LB	0.50 Hz
NUCLEUS	13C	GB	0
D11	0.0300000 sec	PC	1.40
P31	100.0 usec		
S2	20 dB	10 NMR plot parameters	
HL1	1 dB	CX	33.00 cm
D1	1.0000000 sec	F1P	123.000 ppm
P1	6.5 usec	F1	12367.47 Hz
DE	26.3 usec	F2P	-6.000 ppm
SF01	100.5597430 MHz	F2	-603.29 Hz
SWH	23809.58 Hz	PPMCM	3.90909 ppm/cm
TD	65536	HZCM	393.05334 Hz/cm
NS	8000		
DS	2		

The result of the analysis is shown in Fig. 5. The pattern of NMR peaks obtained is the same as it is obtained for levan as published by Gross et al., 1992, Physiol Mol Plant Pathol 40: 371.

This proves that the transformed plants synthesise levan after transformation by one of the constructs described in examples 1 to 3.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Institut für Genbiologische
Forschung Berlin GmbH
Ihnestr. 63
W-1000 Berlin 33

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Institut für Genbiologische Forschung Berlin GmbH Address: Ihnestr. 63 W-1000 Berlin 33	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 7186 Date of the deposit or of the transfer[1]: 1992-07-16

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 1992-07-17 .[2]
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1 B D-3300 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s): U. Weiss Date: 1992-07-21

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

[4] Fill in if the information has been requested and if the results of the test were negative.

BUDAPESTER VERTRAG ÜBER DIE INTERNATIONALE ANERKENNUNG DER HINTERLEGUNG VON MIKROORGANISMEN FÜR DIE ZWECKE VON PATENTVERFAHREN

INTERNATIONALES FORMBLATT

Institut für Genbiologische
Forschung Berlin GmbH
Ihnestr. 63
W-1000 Berlin 33

EMPFANGSBESTÄTIGUNG BEI ERSTHINTERLEGUNG, ausgestellt gemäß Regel 7.1 von der unten angegebenen INTERNATIONALEN HINTERLEGUNGSSTELLE

I. KENNZEICHNUNG DES MIKROORGANISMUS	
Vom HINTERLEGER zugeteiltes Bezugszeichen: p33-CW-Lev	Von der INTERNATIONALEN HINTERLEGUNGSSTELLE zugeteilte EINGANGSNUMMER: DSM 7188

II. WISSENSCHAFTLICHE BESCHREIBUNG UND/ODER VORGESCHLAGENE TAXONOMISCHE BEZEICHNUNG

Mit dem unter I. bezeichneten Mikroorganismus wurde

( ) eine wissenschaftliche Beschreibung
( ) eine vorgeschlagene taxonomische Bezeichnung

eingereicht.
(Zutreffendes ankreuzen).

III. EINGANG UND ANNAHME

Diese internationale Hinterlegungsstelle nimmt den unter I bezeichneten Mikroorganismus an, der bei ihr am 1992-07-16 (Datum der Ersthinterlegung)[1] eingegangen ist.

IV. EINGANG DES ANTRAGS AUF UMWANDLUNG

Der unter I bezeichnete Mikroorganismus ist bei dieser Internationalen Hinterlegungsstelle am eingegangen (Datum der Ersthinterlegung) und ein Antrag auf Umwandlung dieser Ersthinterlegung in eine Hinterlegung gemäß Budapester Vertrag ist am eingegangen (Datum des Eingangs des Antrags auf Umwandlung).

V. INTERNATIONALE HINTERLEGUNGSSTELLE

Name: DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Adresse: Mascheroder Weg 1 B D-3300 Braunschweig	Unterschrift(en) der zur Vertretung der internationalen Hinterlegungsstelle befugten Person(en) oder des (der) von ihr ermächtigten Bediensteten: U. Weiss Datum: 1992-07-21

[1] Falls Regel 6.4 Buchstabe d zutrifft, ist dies der Zeitpunkt, zu dem der Status einer internationalen Hinterlegungsstelle erworben worden ist:
Formblatt DSM-BP/4 (einzige Seite) 0291

Claims

1. DNA sequence, on which the sequence of a levan sucrase is located, characterised in that the sequence of the levan sucrase is derived from a gram-negative bacterium of the species *Erwinia amylovora* with the nucleotide sequence (Seq - ID NO 1):

```
GGATCCCCCG GGCTGCAGCG ATCATGGTTA TTTATAAGGG ATTGTTATGT          50
CCTGAAAACC ACACAACAGA ACCAGAGTGA TTTCAAAAAA TAAAAAGCTA         100
TTAATATACA GACCTTCAGC AAGAAGGTAT TCGAAATAAC CTGTGAGGAT
                                                               150
ATTT  ATG TCA GAT
      Met Ser Asp                                              163
TAT AAT TAT AAA CCA ACG CTG TGG ACT CGT GCC GAT GCA TTG AAA
Tyr Asn Tyr Lys Pro Thr Leu Trp Thr Arg Ala Asp Ala Leu Lys    208
  5                 10                  15
GTT CAT GAG GAT GAC CCA ACC ACA ACT CAA CCG GTT ATT GAC ATT    253
Val His Glu Asp Asp Pro Thr Thr Thr Gln Pro Val Ile Asp Ile
 20                  25                  30
GCA TTC CCG GTA ATG AGT GAA GAA GTC TTT ATT TGG GAT ACC ATG
Ala Phe Pro Val Met Ser Glu Glu Val Phe Ile Trp Asp Thr Met    298
 35                  40                  45
CCA TTG CGA GAC TTC GAC GGA GAG ATT ATC TCT GTA AAT GGT TGG
Pro Leu Arg Asp Phe Asp Gly Glu Ile Ile Ser Val Asn Gly Trp    333
 50                  55                  60
TGT ATT ATT TTT ACG CTA ACA GCA GAT CGC AAC ACT GAT AAT CCG    388
Cys Ile Ile Phe Thr Leu Thr Ala Asp Arg Asn Thr Asp Asn Pro
 65                  70                  75
CAA TTC CAG GAT GAA AAT GGC AAT TAT GAT ATT ACT CGT GAC TGG
Gln Phe Gln Asp Glu Asn Gly Asn Tyr Asp Ile Thr Arg Asp Trp    433
 80                  85                  90
GAA GAC AGA CAT GGT CGT GCG CGT ATT TGT TAT TGG TAC TCA CGC    478
Glu Asp Arg His Gly Arg Ala Arg Ile Cys Tyr Trp Tyr Ser Arg
 95                 100                 105
ACC GGT AAA GAC TGG ATT TTT GGC GGT CGG GTA ATG GCC GAA GGT    523
Thr Gly Lys Asp Trp Ile Phe Gly Gly Arg Val Met Ala Glu Gly
110                 115                 120
GTC GCA CCG ACG ACG CGT GAG TGG GCC GGA ACC CCG ATC CTT TTA
Val Ala Pro Thr Thr Arg Glu Trp Ala Gly Thr Pro Ile Leu Leu    568
125                 130                 135
AAC GAT CGG GGC GAT ATT GAC CTG TAT TAT ACC TGT GTC ACT CCG    613
Asn Asp Arg Gly Asp Ile Asp Leu Tyr Tyr Thr Cys Val Thr Pro
140                 145                 150
GGT GCA ACC ATT GCC AAA GTG CGC GGT AAA ATC GTC ACT TCC GAT    658
Gly Ala Thr Ile Ala Lys Val Arg Gly Lys Ile Val Thr Ser Asp
155                 160                 165
CAA AGT GTA AGC CTG GAA GGT TTT CAG CAG GTT ACA TCA CTT TTC    703
Gln Ser Val Ser Leu Glu Gly Phe Gln Gln Val Thr Ser Leu Phe
170                 175                 180
```

```
TCT GCT GAC GGG ACT ATT TAC CAG ACG GAA GAG CAG AAC GCT TTC 748
Ser Ala Asp Gly Thr Ile Tyr Gln Thr Glu Glu Gln Asn Ala Phe
    185                 190                 195
TGG AAC TTC CGT GAC CCA AGC CCA TTC ATT GAC AGG AAT GAT GGC
Trp Asn Phe Arg Asp Pro Ser Pro Phe Ile Asp Arg Asn Asp Gly 793
    200                 205                 210
AAA TTA TAT ATG CTG TTT GAA GGA AAC GTG GCG GGG CCG CGC GGT
Lys Leu Tyr Met Leu Phe Glu Gly Asn Val Ala Gly Pro Arg Gly 838
    215                 220                 225
TCG CAC GAA ATT ACC CAG GCT GAG ATG GGT AAT GTG CCG CCG GGT 883
Ser His Glu Ile Thr Gln Ala Glu Met Gly Asn Val Pro Pro Gly
    230                 235                 240
TAT GAA GAT GTG GGT GGC GCA AAA TAT CAG GCA GGC TGT GTT GGT
Tyr Glu Asp Val Gly Gly Ala Lys Tyr Gln Ala Gly Cys Val Gly 928
    245                 250                 255
CTG GCT GTG GCC AAA GAC CTG TCA GGC AGT GAG TGG CAA ATC CTG
Leu Ala Val Ala Lys Asp Leu Ser Gly Ser Glu Trp Gln Ile Leu 973
    260                 265                 270
CCT CCG CTG ATC ACC GCT GTT GGC GTA AAC GAT CAG ACT GAA CGC 1018
Pro Pro Leu Ile Thr Ala Val Gly Val Asn Asp Gln Thr Glu Arg
    275                 280                 285
CCT CAT TTT GTC TTC CAG GAT GGT AAA TAC TAT CTG TTC ACC ATT
Pro His Phe Val Phe Gln Asp Gly Lys Tyr Tyr Leu Phe Thr Ile 1063
    290                 295                 300
AGC CAT AAG TAC ACT TTT GCC GAT AAC CTG ACC GGC CCT GAT GGA 1108
Ser His Lys Tyr Thr Phe Ala Asp Asn Leu Thr Gly Pro Asp Gly
    305                 310                 315
GTG TAT GGC TTT GTA AGC GAT AAA CTT ACC GGC CCT TAC ACG CCG
Val Tyr Gly Phe Val Ser Asp Lys Leu Thr Gly Pro Tyr Thr Pro 1153
    320                 325                 330
ATG AAT AGC TCC GGG CTG GTG CTG GGC AAC CCG TCT TCA CAA CCT 1198
Met Asn Ser Ser Gly Leu Val Leu Gly Asn Pro Ser Ser Gln Pro
    335                 340                 345
TTC CAG ACA TAT TCA CAC TAT GTT ATG CCT AAT GGG CTG GTC ACT 1243
Phe Gln Thr Tyr Ser His Tyr Val Met Pro Asn Gly Leu Val Thr
    350                 355                 360
TCC TTT ATT GAC AGT GTT CCG TGG AAA GGT AAG GAC TAT CGC ATT
Ser Phe Ile Asp Ser Val Pro Trp Lys Gly Lys Asp Tyr Arg Ile 1288
    365                 370                 375
GGC GGT ACT GAA GCT CCG ACC GTA AAA ATT CTG TTG AAA GGC GAT 1333
Gly Gly Thr Glu Ala Pro Thr Val Lys Ile Leu Leu Lys Gly Asp
    380                 385                 390
CGC TCA TTT ATT GTT GAT AGC TTC GAT TAT GGA TAT ATT CCG GCA 1378
Arg Ser Phe Ile Val Asp Ser Phe Asp Tyr Gly Tyr Ile Pro Ala
    395                 400                 405
ATG AAA GAC ATT ACT TTA AAA TAAGTCTGTT GTCGATATCA AGCTTATCGA 1429
Met Lys Asp Ile Thr Leu Lys
    410                 415
TACCGTCGA 1438
```

whereby by integration of this sequence in a plant genome, polyfructan (levan) expression in leaves and tubers is made possible.

2. Plasmids containing a DNA sequence according to claim 1, for the preparation of plants with polyfructan (levan) expression in leaves and tubers.

3. Plasmid p35s-CW-LEV (DSM 7186).

4. Plasmid p35s-CY-LEV (DSM 7187).

5. Plasmid p33-CW-LEV (DSM 7188).

6. Process for the preparation of transgenic plants with polyfructan (levan) expression in leaves and tubers, which comprises the following steps:
   (a) preparation of a DNA sequence with the following partial sequences:
      i) a promoter which is active in plants and ensures formation of an RNA in the intended target tissues or target cells,
      ii) DNA sequence of a levan sucrase according to claim 1, and
      iii) a 3'-non-translated sequence, which in plant cells leads to the termination of the transcription as well as the addition of poly A residues to the 3'-end of the RNA,
   (b) transfer and integration of the DNA sequence in the plant genome of a recombinant double stranded DNA molecule from plant cells using a plasmid according to any one of claims 2 to 5, and
   (c) regeneration of intact whole plants from the transformed plant cells.

7. Process according to claim 6, characterised in that the polyfructan (levan) sucrase expression in leaves and tubers leads to a polyfructan (levan) sucrase

activity in the chloroplasts, amyloplasts, mitochondria, cytosol, inter cellular space and/or vacuoles.

8. A plant obtained by the process according to claims 6 or 7.

9. A plant according to claim 8, which is crop plant.

10. A plant according to claim 8 or 9, which is a maize, rice, wheat, barley, sugar beet, sugar cane, tobacco or potato plant.

11. Use of the DNA sequence according to claim 1, for the preparation of plants with polyfructan (levan) expression in leaves and tubers.

12. Use of the plasmids according to any one of claim 2 to 5, for the preparation of plants with polyfructan (levan) expression in leaves and tubers.

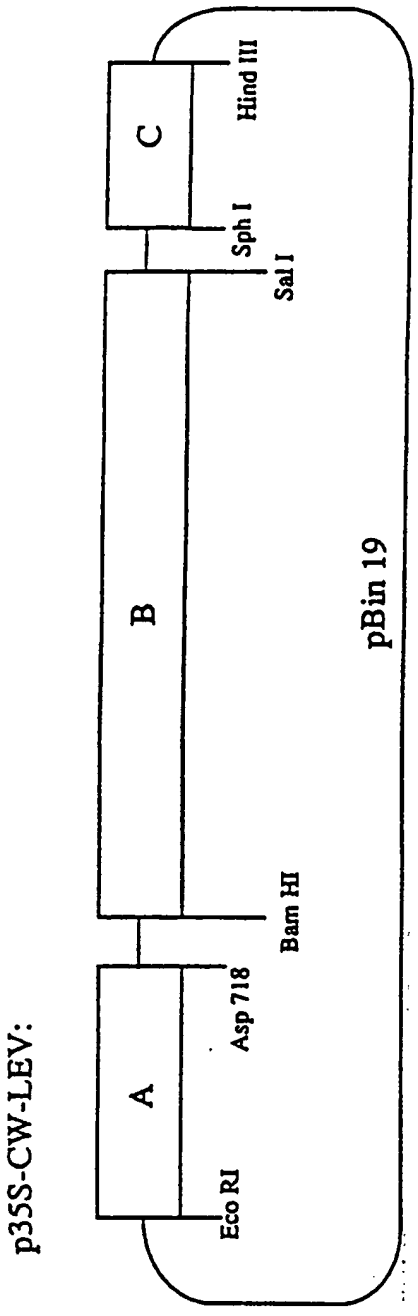

Fig. 1

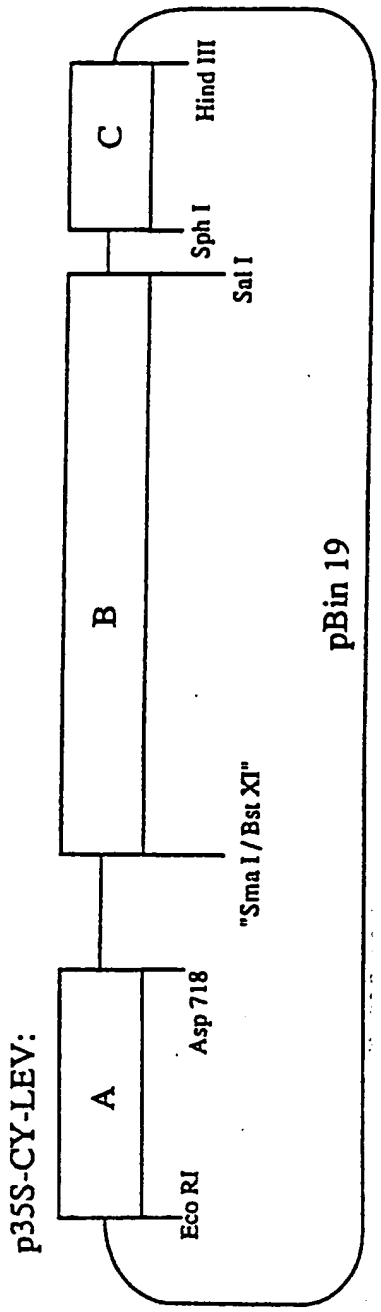
p35S-CY-LEV:
A
B
C
Eco RI
Asp 718
"Sma I / Bst XI"
Sal I
Sph I
Hind III
pBin 19

Fig. 2

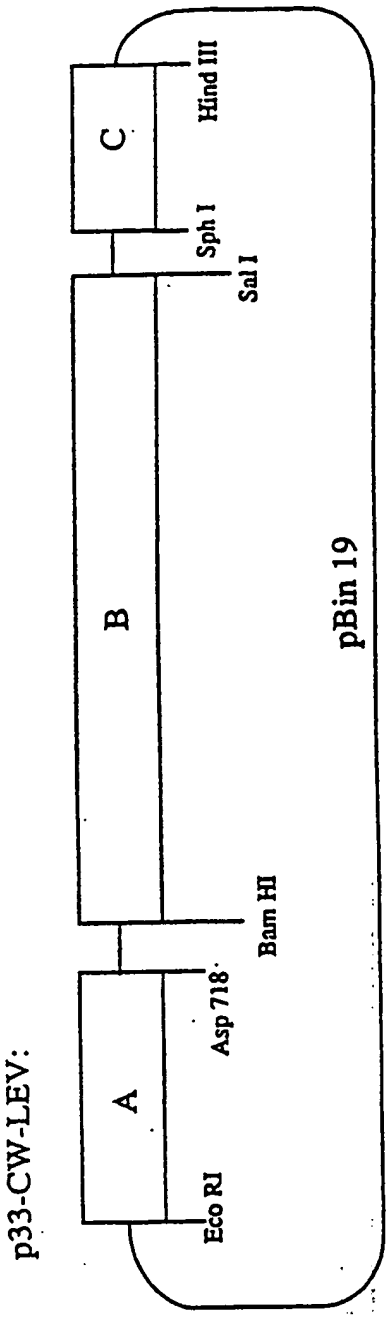

Fig. 3

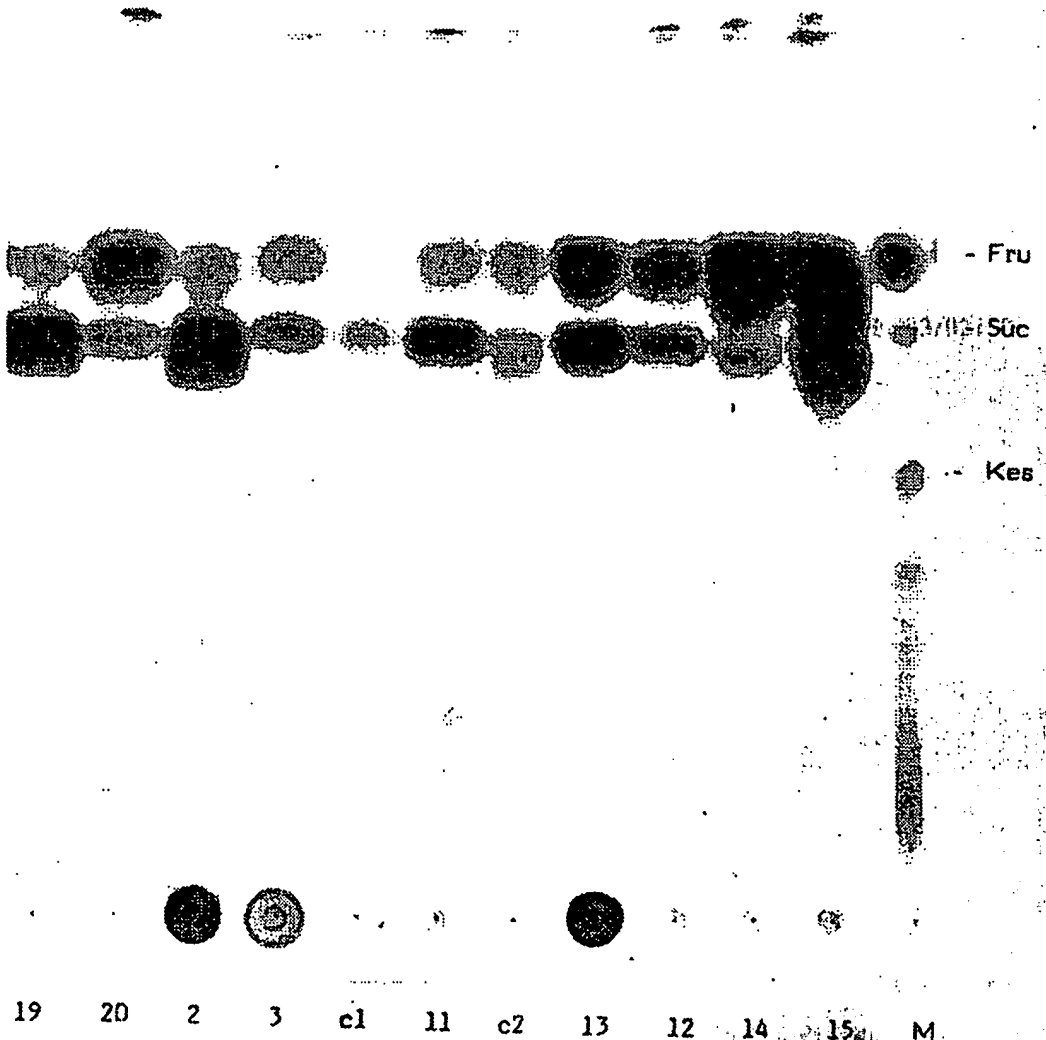

Fig. 4

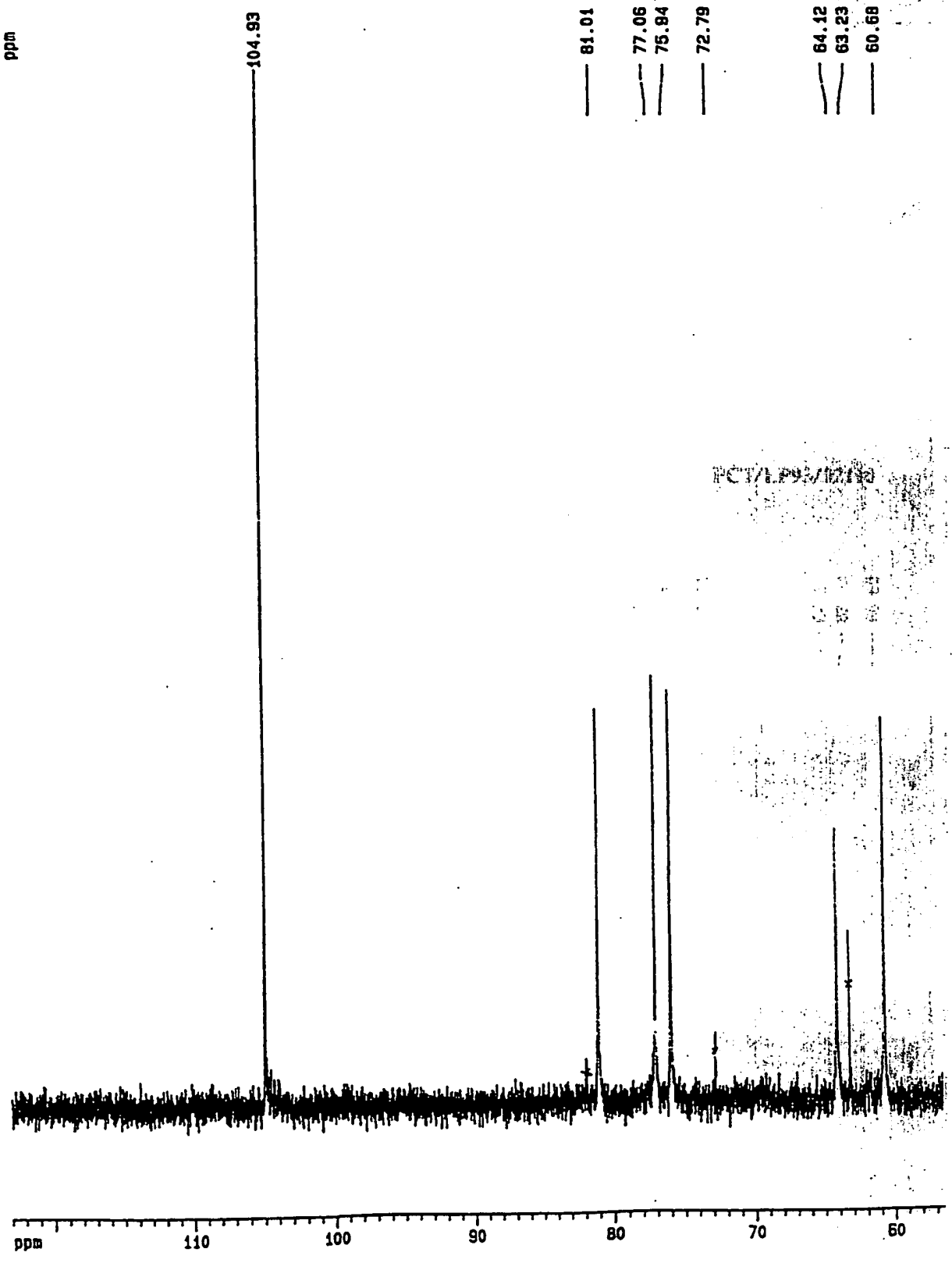
ppm
104.93
81.01
77.06
75.84
72.79
64.12
63.23
60.68
ppm
110
100
90
80
70
60

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 93/02110

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/82 C12N15/54 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	MITTEILUNGEN AUS DER BIOLOGISCHEN BUNDESANSTALT FUER LAND- UND FORSTWIRTSCHAFT BERLIN-DAHLEM(COMUNICATIONS FROM THE FEDERAL BIOLOGICAL INSTITUTE FOR AGRICULTURE AND FORESTRY BERLIN-DAHLEM); SYMPOSIUM ON FIREBLIGHT, LADENBURG, GERMANY, JUNE 13-14,1991 vol. 282 , 1992 pages 78 - 81 GEIER, G., ET AL. 'Levansucrase as a virulence factor in fireblight development' see page 80, paragraph 3 -paragraph 5 ---	1,2
A	WO,A,89 12386 (CALGENE) 28 December 1989 cited in the application see example 4 ---	1-12

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 1993	10 -12- 1993

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	MADDOX, A

Form PCT/ISA/210 (second sheet) (July 1992)

1-12

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 93/02110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CARBOHYDRATE RESEARCH vol. 190 , 1989 pages 299 - 307 COTE, G.L., ET AL. 'Purification and properties of an extracellular levansucrase from Erwinia herbicola NRRL B-1678' see the whole document ---	1-12

International Application No
PCT/EP 93/02110

1

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/EP 93/02110

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8912386	28-12-89	AU-A- 3852089	12-01-90

International Application No
PCT/EP 93/02110